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In vivo binding of carbon monoxide to cytochrome *c* oxidase in rat brain

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BROWN, S. D., AND C. A. PIANTADOSI. *In vivo binding of carbon monoxide to cytochrome c oxidase in rat brain.* J. Appl. Physiol. 68(2): 604–610, 1990.—The possibility of binding of CO to cytochrome *c* oxidase (cytochrome a,a_3) in brain cortex has been examined in vivo by reflectance spectrophotometry. During ventilation with CO-containing gases, cytochrome a,a_3 absorption at 605 nm increased in the parietal cortex of anesthetized rats during carboxyhemoglobin (HbCO) formation. HbCO levels, measured by changes in absorption at 569–586 nm in vivo, correlated positively with arterial HbCO by CO oximetry. Arterial blood pressure and calculated O_2 content varied inversely with HbCO. During CO exposure, decreases in blood pressure, O_2 content, and cytochrome a,a_3 oxidation level could be reversed partly at constant HbCO by compression to 3 atmospheres absolute (ATA). After removing CO from inspired gas at 3 ATA, optical and physiological parameters recovered completely to control values except for minor persistent elevations of HbCO. Difference spectra from parallel experiments at constant HbCO revealed absorption minima at 588–592 nm and 600–605 nm as a result of hyperbaric exposure. Spectral analysis of these components was consistent with partial dissociation of a cytochrome a_3-CO complex and cytochrome *a* reoxidation with increasing dissolved O_2 in hyperbaric conditions.

carboxyhemoglobin; cytochrome a,a_3; hyperbaric oxygen; hypoxia; reflectance spectrophotometry

THE CLASSIC MECHANISM of CO toxicity is tight binding of CO to hemoglobin, which decreases the O_2-carrying capacity of arterial blood, shifts the O_2-hemoglobin dissociation curve to the left, and produces tissue hypoxia (3, 24). Intracellular effects, such as CO binding to compounds like myoglobin and cytochrome *c* oxidase, have been long recognized in vitro but are of unknown physiological significance (8, 17). The toxic effects of CO do not all appear to fit the classic mechanism; thus, some investigators believe that there is a direct tissue effect of CO. In vivo binding of CO to cytochrome *c* oxidase has not been previously demonstrated in the presence of carboxyhemoglobin (HbCO). The purposes of the present study were threefold: *1*) to investigate in vivo oxidation-reduction (redox) responses of cytochrome *c* oxidase induced by CO hypoxia, *2*) to detect the possible formation and reversal of the cytochrome a_3-CO ligand in the rat brain, and *3*) to correlate those findings with the cardiovascular responses, measured HbCO, and arterial pH and blood gas values. Our data indicate that CO hypoxia increases cytochrome *c* oxidase reduction levels and binds to reduced oxidase species in the brain. These cerebrocortical effects were partly reversible by hyperbaric oxygenation at constant HbCO concentrations. A preliminary report of these findings has been presented (6).

METHODS

There were three parts to the experiment. First, CO mediated cardiopulmonary and cerebrocortical cytochrome a,a_3 responses to 0.5 or 1.0% CO, and their changes with hyperbaric oxygen were measured in vivo. Second, HbCO formation assessed spectrophotometrically in vivo was correlated with measured HbCO in a series of graded CO exposures. Third, in vivo spectroscopic scans were made to determine whether CO-mediated cytochrome a,a_3 responses were due only to an increase in the reduction state of cytochrome *a* during CO hypoxia or to both hypoxia-related reduction responses and formation of the cytochrome a_3-CO complex.

Animal preparation. Adult male Sprague Dawley rats (Charles Rivers Laboratories) weighing 150–300 g were used in the studies. The rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and tracheostomies were performed. Polyethylene catheters were placed in both femoral arteries and one femoral vein. The animals were transferred to a large hyperbaric chamber within the F. G. Hall Hypo-Hyperbaric Center. The rats were paralyzed with tubocurarine chloride (1.5 mg/kg iv) to prevent respiratory motion and ventilated with 90% O_2–10% N_2 via a mechanical rodent ventilator (EDCO Scientific, Chapel Hill, NC). Additional pentobarbital and curare were given intravenously as necessary to maintain anesthesia and immobilization. The head was secured in a stereotaxic apparatus and the skull exposed via a longitudinal incision through the scalp. The muscle and fascia were reflected from a point anterior of the nasal suture to well behind the parietooccipital sutures. The rat's arterial blood pressure (Statham, model 23d, strain gauge), bipolar electroencephalogram (EEG; Grass Instruments, platinum needle electrodes), and rectal temperature were monitored continuously throughout the experiments. A thermostatically controlled heating pad beneath the rat maintained core temperature near 37°C. Arterial blood samples were drawn intermittently for measurement of arterial Po_2, Pco_2, and pH (Pa_{O_2}, Pa_{CO_2}, and pH_a; Instrumentation Laboratories, model 813 pH/

blood gas analyzer). Respiratory rate and/or tidal volume were adjusted to maintain Pa_{CO_2} near 35 Torr. Additional aliquots of arterial blood (200 μl) were used to measure total hemoglobin in grams per 100 ml blood, percent oxyhemoglobin (HbO_2), percent HbCO, percent deoxyhemoglobin, percent methemoglobin, and initial O_2 content (Instrumentation Laboratories, model 482 CO-oximeter) (5). A total of four 0.75-ml aliquots of blood was removed for these analyses. One milliliter of normal saline was infused intravenously after removal of each blood sample. Blood gas analysis was performed in the chamber at 3 atmospheres absolute (ATA) by a second blood gas instrument (Radiometer, Copenhagen) modified to operate at pressure. Arterial blood samples obtained at 3 ATA for CO oximetry were sealed at 0°C and analyzed after decompression. O_2 content as milliliters O_2 per 100 ml blood (vol%) to include dissolved O_2 volume was calculated using the following equation

$$\text{total } O_2 \text{ vol\%} = \{[\text{measured } Pa_{O_2} \text{ (Torr)}] \times [0.003 \text{ ml dissolved } O_2/\text{Torr}]\} + (O_2 \text{ content from CO oximetry})$$

Optical monitoring. The parietal cortex of the rat was monitored continuously by differential reflectance spectrophotometry. The optical measurements were made through the translucent intact skull with a four-wavelength spectrophotometer. This monitoring approach is possible in small rats because the skull is thin (<1 mm) and translucent (20). Transcranial monitoring maintains normal intracranial circulatory relationships at the expense of some light scattering. Such scattering may decrease the optical signal-to-noise ratio; however, direct optical coupling of a light guide to the skull to collect photons also tends to minimize artifacts from specular reflection. The spectrophotometer was of a type described by Jöbsis et al. (16) wherein a single incandescent light source (Osram model P35s) supplies four tunable monochromators (Instruments SA, model H-10 and H-10V) to produce two pairs of monochromatic sample and reference wavelengths. The spectral half bandwidth was 3 nm at 620 and 605 nm and 4 nm at 586 and 569 nm. The light was pulsed by means of a slotted chopping wheel and was conducted to the skull surface via a fiber-optic bundle. At the tip of the bundle, an internally reflecting glass rod illuminated a small area of the rat's skull. The depth of penetration of visible wavelengths of incident light into the cortical cell layers is probably no more than 1 mm, based on nearly logarithmic light absorption by biologic tissues and relative intensities of transmission and reflectance spectra measured through intact skull (20). A similar rod with an opaque sheath coupled to the other parietal bone with optical coupling gel (Math Associates, Westbury, NY) collected diffusely reflected light to be measured by a side-window photomultiplier tube (Hamamatsu R928). A neoprene O ring at the junction of the collecting rod with the skull excluded extraneous light. Differences in intensity of sample and reference wavelengths were recorded on a multichannel chart recorder (Gould, model 560).

The difference spectrum of cytochrome a,a_3 has an absorption maximum at ~605 nm. About 80% of this absorption peak is derived primarily from the reduced iron-porphyrin complex of cytochrome *a*, whereas only 20% of the absorption at 605 nm is attributable to reduced cytochrome a_3 (17, 29). Changes in absorption at the 605-nm sample wavelength were corrected for light scattering by subtracting changes at a reference wavelength of 620 nm (20). HbCO was monitored optically at the 569- to 586-nm wavelength pair. The latter two wavelengths are isosbestic points for hemoglobin (Hb) and HbO_2, and 569 nm is an absorption peak for HbCO (30). Contribution to the 605- to 620-nm wavelength pair from hemoglobin is ~15%; contributions from other cytochromes are minor and have been neglected for the purposes of this study (27). Analysis of these contributions was not possible with the dual-wavelength approach, but this was not a significant shortcoming because of the qualitative nature of the experiments. Optical data were expressed as a percent of the total labile signal (TLS) measured at each wavelength pair. The TLS for 605–620 nm was defined as the difference between maximal oxidation at 1 or 3 ATA and reduction at death. The TLS for 569–586 nm was defined as the difference between control conditions and values obtained while the rat was ventilated with either 1.0 or 0.5% CO in 90% O_2-10% N_2.

CO and hyperbaric exposures. The spectrophotometer was installed in the hyperbaric chamber, and potential sources of combustion were flushed continuously with N_2 during the experiment. Electrical cables from the spectrophotometer to the signal-conditioning amplifier transversed the hull via connectionless epoxy-potted penetrations. A schematic diagram of the experimental setup is provided in Fig. 1. At the beginning of the experiment, arterial blood samples were obtained for control determinations of blood gases, pH, and CO oximetry while the animal breathed 90% O_2. The rat was then ventilated with 90% O_2 and either 1.0% CO for 15 min (or until optical stability) or 0.5% CO for 30 min. CO oximetry readings were repeated at the end of each CO exposure period before the chamber was compressed to 3 ATA. The rate of compression was 0.30 ATA/min. During compression the animal was continuously ventilated with the CO mixture. Arterial blood gas analysis and CO oximetry readings were repeated 20 min after compression began. The animals were then ventilated

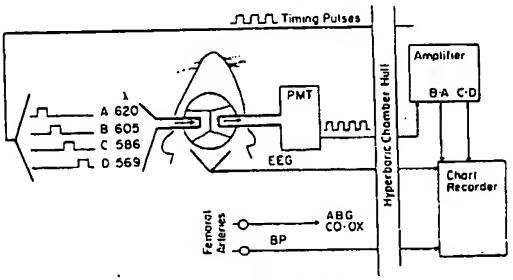

FIG. 1. Experimental setup for physiological and optical monitoring in a hyperbaric chamber. BP, blood pressure; ABG, arterial blood gas.

with 90% O_2-10% N_2 for 25 min, and blood sampling was repeated for gas analysis and CO oximetry measurements. Rats were killed then with an overdose of intravenous KCl and the 605- to 620-nm wavelength pair was observed until stable before chamber decompression. Preliminary experiments demonstrated steady-state optical signals and HbCO levels within the chosen time periods.

In vivo HbCO formation and cytochrome a,a_3 redox state during graded CO exposures were assessed optically and correlated with HbCO measured by CO oximetry. Six rats were monitored as described above during ventilation with 90% O_2 and 0.25% CO for 30 min, then 0.5% CO for 20 min, and finally 1.0% CO for 15 min. Optical signals and HbCO levels were stable at the end of the designated periods. The rats were killed with intravenous KCl after the 1.0% CO exposure period to obtain the 605- to 620-nm TLS.

In vivo absorption spectra were recorded to detect the cytochrome a_3-CO complex in eight rats. Spectral scans were obtained by recording transmittance values in 4-nm increments over the wavelength range between 620 and 568 nm by means of a single monochromator at 4-nm spectral bandwidth. Spectra were obtained at optical steady states after *1*) ventilation with 90% O_2 and either 1.0% CO for 15 min ($n = 4$) or 0.5% CO for 30 min ($n = 4$) at 1 ATA, *2*) 12 min after compression began to 3 ATA, and *3*) 8 min after death at 3 ATA. Transmittance values from consecutive scans were converted to log ratios to obtain absorption difference spectra between oxidized and reduced conditions. These rats underwent the same procedures for physiological monitoring and blood sampling as all other animals.

Statistical methods. Grouped data were expressed as means ± SE. Group-to-group comparisons were made by unpaired *t* tests. Statistical comparisons for control and experimental data from the same animal were made by paired *t* test or one-way analysis of variance. Multiple comparisons were made using Bonferroni corrections. $P < 0.05$ was accepted as significant. Absorption difference spectra were generated by averaging optical density differences at specific wavelengths and fitting smooth curves to the data as open Q-splines (Statgraphics 2.6, Statistical Graphics, Rockville, MD).

RESULTS

The physiological and optical parameters measured at control conditions, after CO exposure at 1 and 3 ATA, and after 90% O_2 at 3 ATA are summarized in Table 1. Mean arterial pressure (MAP) decreased in all animals but stabilized in ~9 min after exposure to 90% O_2-1% CO-balance N_2 and in ~15 min after exposure to 90% O_2-0.5% CO. MAP increased after compression to 3 ATA and stabilized in ~8 min in rats breathing the 1% CO gas mixture and in ~9 min in rats breathing the 0.5% CO gas mixture. Further treatment of both groups with 90% O_2 at 3 ATA for 25 min resulted in recovery of the above parameters to at least control values except for slight amounts of residual HbCO.

A representative experimental trace from a rat exposed to 1% CO is shown in Fig. 2. The optical HbCO signal indicated rapid uptake of CO, but the signal remained stable after equilibration, despite a decrease in MAP and reduction of cytochrome a,a_3 during CO exposure. These trends were reflected by rats in both groups where absorption increased with 1 and 0.5% CO at 569 nm relative to 586 nm (the optical HbCO signal). The HbCO signal change began after ~1 min and stabilized after ~20 min of exposure in both groups. At 605 relative to 620 nm, absorption increased consistent with cytochrome *a* reduction after ~2.5 min of CO exposure in both groups and stabilized in ~9 and 23 min in the 1 and 0.5% CO groups, respectively. The EEG signal attenuated in 8 of 10 rats in the 1% CO group and 1 of 10 animals in the 0.5% CO group after exposure.

With compression to 3 ATA, absorption at 605 nm

TABLE 1. *General physiological and optical variables*

	1% CO-90% O_2-Exposed Rats				0.5% CO-90% O_2-Exposed Rats			
	Control	1 ATA + CO	3 ATA + CO	3 ATA	Control	1 ATA + CO	3 ATA + CO	3 ATA
MAP, mmHg	104±7 (10)	62±6* (10)	93±8† (10)	108±8† (10)	92±4 (10)	75±4* (10)	98±6† (10)	104±6* (10)
Temp,‡ °C	37.6±0.1 (9)	37.6±0.2 (9)	38.0±0.3 (9)	37.7±0.3 (9)	37.0±0.3 (10)	37.2±0.2 (10)	37.5±0.2 (10)	37.3±0.2 (10)
pH_a‡	7.43±0.02 (10)	7.34±0.03 (6)	7.38±0.02 (8)	7.38±0.03 (9)	7.44±0.02 (9)		7.42±0.01 (10)	7.46±0.02 (10)
Pa_{CO_2},‡ Torr	33.8±1.6 (10)	29.0±4.2 (2)	33.6±1.0 (8)	37.2±2.5 (9)	29.4±2.0 (9)		27.0±2.3 (10)	26.3±2.4 (10)
Pa_{O_2},‡ Torr	346±24 (10)	410 (1)	1,315±55* (9)	1,265±65* (9)	363±11 (9)		1,246±77* (10)	1,467±23* (10)
Ca_{O_2}, vol%	19.3±0.6 (8)	6.7±0.2* (8)	9.7±0.3*† (8)	19.7±0.7*† (8)	17.1±0.6 (10)	8.9±0.4* (10)	12.0±0.5*† (10)	20.2±0.8*† (10)
HbCO, %	3.3±0.1 (8)	69.0±0.7* (6)	69.6±0.7* (8)	8.2±1.2*† (8)	3.0±0.3 (10)	53.1±0.7* (10)	52.4±1.0* (10)	5.7±0.6*† (10)
a,a_3, %TLS	87.0±4.2 (10)	38.3±3.5* (10)	78.7±4.2† (10)	99.8±0.2† (10)	90.8±3.8 (8)	50.9±4.7* (8)	84.9±3.0† (8)	97.3±2.1† (8)
HbCO, %TLS	0.0±0.0 (10)	91.5±4.2* (10)	91.9±4.7* (10)	−16.2±8.9† (10)	0.0±0.0 (8)	85.0±9.1* (8)	94.3±4.6* (8)	−7.8±9.5† (8)

Values are means ± SE of no. of rats shown in parentheses. MAP, mean arterial pressure; pH_a, Pa_{CO_2}, Pa_{O_2}, arterial pH, PCO_2, PO_2; Ca_{O_2}, arterial O_2 content; HbCO, carboxyhemoglobin; TLS, total labile signal. * $P < 0.05$ compared with control; † $P < 0.05$ compared with previous condition; ‡ no significant differences within groups by analysis of variance.

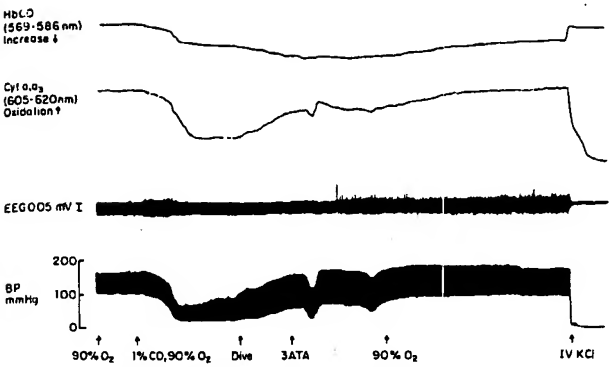

FIG. 2. Continuous differential optical recordings of effects of 1% CO in 90% O_2 at 1 and 3 ATA on cerebral cytochrome a,a_3 and HbCO formation compared with EEG and blood pressure.

decreased rapidly relative to 620 nm (cytochrome *a* reoxidation), whereas the 569- to 586-nm signal did not change in either CO exposure group. The latter observation was confirmed by no change in measured HbCO before and 20 min after compression ($P > 0.90$ and 0.40 for 1 and 0.5% CO). During 1 and 0.5% CO exposure at 3 ATA, absorption at 605 nm decreased to 79 ± 4% of the TLS in 6.8 ± 1.4 min and 86 ± 3% of the TLS in 9.3 ± 1.3 min after compression began, respectively. After compression to 3 ATA, the EEG recovered within 10 min in all rats. Further treatment of both groups with 90% O_2 at 3 ATA for 25 min recovered the 605- to 620-nm wavelength pair to control values. Negative values at 569–586 nm after recovery indicate that the mean optical values after recovery exceeded control values that were defined as zero.

Graded CO exposure. HbCO formation assessed spectrophotometrically in vivo was correlated with measured HbCO in a series of graded CO exposures. The relationship between measured HbCO and absorption changes at the 569- to 586-nm and 605- to 620-nm wavelength pairs in graded 0.25, 0.5, and 1.0% CO exposures was linear with a high degree of correlation (Fig. 3). Rats exposed to the graded CO concentrations had less cytochrome a,a_3 reduction on 1.0 and 0.5% CO than rats exposed acutely to the same CO concentrations ($P <$ 0.05 for 1 and 0.5% CO).

In vivo absorption spectra. Absorption difference spectra comparing CO exposures at 1 ATA (reduced) with 3 ATA (oxidized) and CO exposures at 3 ATA (oxidized) with death at 3 ATA (reduced) are shown in Fig. 4, which represents mean absorption values from four experiments at each CO concentration. The 3 ATA-1 ATA data were expressed as oxidized minus reduced for ease of comparison with the reduced minus oxidized spectra recorded at death. Of note, the 3 ATA oxidized minus reduced on CO spectra had two absorption minima. The shorter wavelength absorption component was found at 588–590 nm, whereas the longer wavelength component peak was located at 600–604 nm. The latter component also was more prominent in the 1% CO-exposed rats. These absorption characteristics are consistent with dissociation of the cytochrome a,a_3-CO ligand and reoxidation of cytochrome a,a_3. The reduced at death minus oxidized at 3 ATA spectra demonstrates the expected single peak of cytochrome a,a_3 reduction at ~605 nm. Subtraction of reduced minus oxidized spectra at death from oxidized minus reduced on CO spectra in Fig. 4

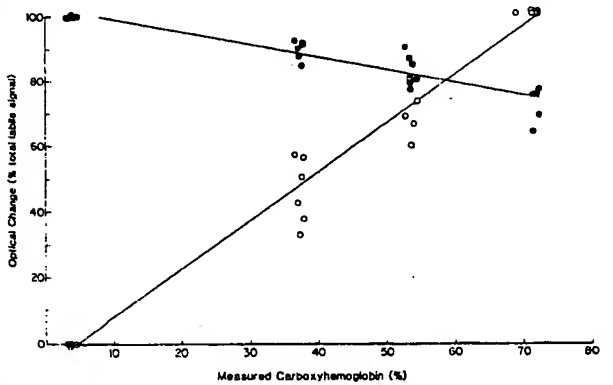

FIG. 3. Correlation of changes in 569- to 586-nm (HbCO, ○, $r^2 = 0.96$) and 605- to 620-nm (cytochrome a,a_3, ●, $r^2 = 0.86$) wavelength pairs to HbCO measured by CO oximetry during graded 0.25, 0.5, and 1% CO in 90% O_2 exposures.

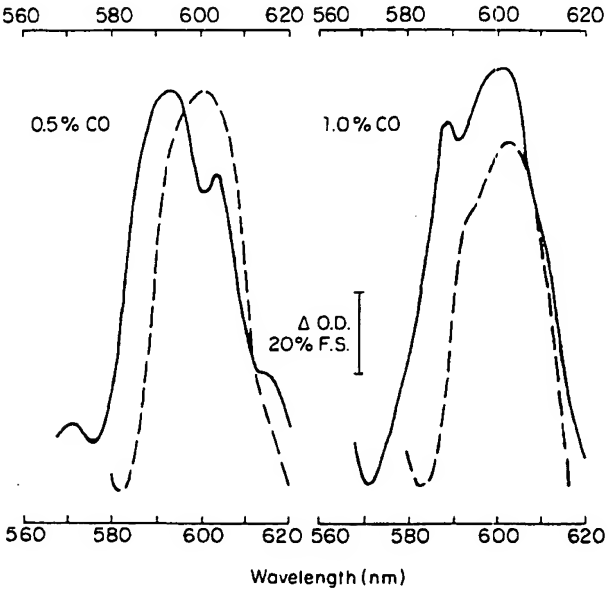

FIG. 4. Absorption difference spectra comparing oxidized at 3 ATA with reduced at 1 ATA (solid lines) and reduced at death (dashed lines) during 1 and 0.5% CO in 90% O_2 exposures. Each spectrum is mean of 4 experiments. FS, full scale.

produced two difference spectra shown in Fig. 5. These spectra confirm that the optical difference between CO hypoxia and simple ischemic hypoxia consists of well-defined absorptions at 586–588 nm with accompanying loss of optical density at 600–605 nm consistent with formation of the cytochrome a_3-CO complex from reduced cytochrome c oxidase (17, 29, 31).

DISCUSSION

Binding of CO to mammalian cytochrome a,a_3 has been recognized since 1939, when Keilin and Hartree (17) measured the reduced cytochrome a_3-CO complex in the porcine heart. The interaction of CO with cytochrome a,a_3 in the presence of circulating hemoglobin, however, has not been reported previously. Most studies of cytochrome a,a_3 and CO have used isolated cytochrome c oxidase or mitochondrial preparations in vitro (7, 17, 31, 33). Some studies, including in vitro observations conducted under nonphysiological conditons, cast doubts about the physiological occurrence of the cytochrome a,a_3-CO interaction (2, 14, 31). Effects of CO have been reported, however, under conditions where hypoxia alone does not appear to account for the responses (10, 13, 15, 32).

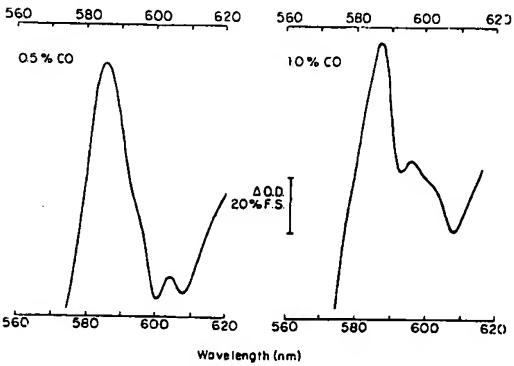

FIG. 5. Subtraction of paired absorption difference spectra from Fig. 4, demonstrating a 585- to 590-nm absorption peak consistent with the cytochrome a_3-CO complex during 1 and 0.5% CO in 90% O_2 exposures.

CO hypoxia produces certain physiological responses, such as hypotension, that would be expected to decrease tissue P_{O_2} and favor CO uptake by cytochrome a,a_3 in vivo. In our rats, CO produced dose-dependent hypotension. The mechanism of this hypotensive response is unclear, although earlier studies of acute CO toxicity that results in HbCO levels of 30–40% also demonstrated a fall in MAP (12, 23, 25) and systemic vasodilation (26), despite increases in cardiac output (1). One study did not report hypotension when awake adult and newborn sheep were titrated slowly to a HbCO level of 55%; however, the cerebral metabolic rate for O_2 decreased in the adult animals (18). Two groups of investigators removed the hypoxic effect of CO toxicity by perfusion with fluorocarbon emulsion and still found decreases in blood pressure (21, 28). Barbiturate anesthesia also may potentiate the hypotensive effect of CO (23).

Regardless of the mechanism, CO-induced hypotension will eventually produce tissue hypoxia through de-

creased perfusion. Ordinarily, the affinity of CO for hemoglobin is at least 50 times that of cytochrome oxidase (34); however, hypotension (9) and arterial hypoxemia (19) both shift CO from the blood to the tissue compartment. The common mechanism is decreased tissue Po_2 shown experimentally to be associated with increased tissue uptake of CO (11). The CO-induced leftward shift of the oxyhemoglobin dissociation curve further exacerbates the tissue hypoxia associated with hypotension or hypoxemia and therefore accentuates CO uptake by tissue (11, 24). In our studies, CO hypoxia was accompanied by cytochrome a,a_3 reduction responses in the brain soon after the onset of CO exposure. These responses may have been exacerbated by decreased cerebral perfusion caused by the hypotension.

The brain is particularly vulnerable to the effects of CO hypoxia. In anesthetized brain cortex under normoxic conditions, intramitochondrial cytochrome c oxidase is partly reduced (27). The oxidase is known to bind CO in any one of its four reduced states in vitro, and each CO compound has its own spectrum. These spectra demonstrate relative persistence of the 590-nm peak of the cytochrome a_3-CO ligand, whereas the 605-nm peak, contributed primarily by the reduced heme moiety of cytochrome a, diminishes with progressive oxidation of the enzyme (33).

In our studies, increases in the reduction level of cytochrome oxidase mediated by CO were indicated by increased absorption at 605 nm relative to 620 nm. This finding, however, did not suffice to demonstrate CO binding to the oxidase. Reduction of the oxidase by hypoxia, CO, or electron transport inhibition by CO produces an absorption peak at 605 nm (4, 17). The cytochrome a_3-CO complex produces an additional absorption peak at 585–590 nm (17). The 585- to 590-nm peak has also been observed in vitro during the conversion of CO to CO_2 by the oxidase either in the presence of O_2 (35) or under anaerobic conditions by its combination with water (4). These possibilities necessitated analysis of cerebrocortical cytochrome spectra from the CO-exposed animals.

We obtained cytochrome difference spectra that were relatively free from interference by hemoglobin because there was little deoxyhemoglobin present at the high Po_2 of the study and most of the HbO_2 was converted to HbCO during the CO exposures. The concentration of HbCO and HbO_2 were kept constant during spectral scans. Therefore, when spectra from the 3-ATA CO exposures were subtracted from the 1-ATA CO exposures, absorption peaks at 585–590 and 605 nm disappeared, indicating loss of absorption by the cytochrome a_3-CO complex and reoxidation of reduced cytochrome a,a_3 under hyperbaric conditions. Thus the disappearance of a 585- to 590-nm peak at hyperbaric conditions represents two possible events related to the cytochrome a_3-CO ligand: *1*) reversal of CO binding to cytochrome a_3 and *2*) metabolism of CO to CO_2 by the oxidase. Cytochrome a_3-CO ligand reversal probably occurred under hyperbaric conditions without a change in HbCO, because absolute Po_2 increased at the site of O_2 metabolism, shifting the redox equilibrium away from reduced cytochrome a,a_3.

The formation of HbCO could be followed reliably in our experiments by the simple subtraction of 586 nm from 569 nm. Despite the good correlation between this in vivo measurement and HbCO measured by CO oximetry, other compounds absorb light at those wavelengths. The 569- to 586-nm pair contains an unquantified contribution from cytochromes of the *b* type, which respond in the brain to CO exposure in vivo, as well as a small contribution from cytochromes *c* and c_1 (22). The 569- to 586-nm wavelength pair also neglects the unequal opposite effects of HbCO formation at 586 nm relative to 569 nm. These factors may explain the negative HbCO %TLS values at 3 ATA in Table 1.

Comparison of ratios of oxidized to reduced or CO-bound oxidase between our two groups of CO-exposed rats could not be made because the optical changes were qualitative. However, the higher 590-to-605-nm ratio in the 0.5% CO group suggests that those rats had a greater proportion of cytochrome a_3-CO ligand compared with reduced cytochrome a,a_3 than the 1% CO group. This unexpected observation could be a result of two factors. First, CO hypoxia was more severe with 1% CO than 0.5% CO and may have been associated with a lower mitochondrial Po_2 and hence a higher reduction level of the cytochrome a component. Second, the 0.5% CO exposure was longer than 1% exposure, thus providing more time for equilibration of CO with reduced cytochrome a_3 components. These explanations and our spectra are consistent with spectra from in vitro studies of the cytochrome a_3-CO interaction at various redox states of the oxidase (33), although we cannot assign precise redox states to the oxidase molecules that contribute to the in vivo spectra.

The intent of these experiments was not to ascertain the physiological significance of CO binding with cytochrome a,a_3 but to demonstrate such binding and its reversal in vivo as a possible explanation for nonhypoxic mechanisms of CO toxicity. The experiments also do not prove the efficacy of hyperbaric O_2 in CO poisoning, although the rationale for its use is strengthened inasmuch as reoxidation of cytochrome a,a_3 and some reversal of CO binding occurs despite constant HbCO level at 3 ATA.

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